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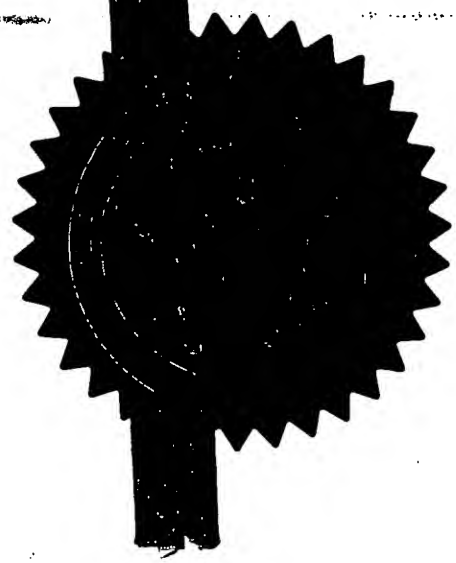

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Signed Stephen Hordley

Dated 8 July 2004

Patents Form 1/77
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1/77

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PPL THERAPEUTICS (SCOTLAND) LIMITED
Roslin
Edinburgh EH25 9PP
UK

Patents ADP number *(if you know it)*

If the applicant is a corporate body, give the country/state of its incorporation

GB 7158578002

4. Title of the invention

METHODS

5. Name of your agent *(if you have one)*

"Address for service" in the United Kingdom to which all correspondence should be sent *(including the postcode)*

KILBURN & STRODE
20 RED LION STREET
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Country	Priority application number *(if you know it)*	Date of filing *(day / month / year)*

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Signature Date

Kilburn & Strode Kilburn & Strode 4.3.99 4 March 1999

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METHODS

This invention describes the production of genetically modified animals in which the genetic modifications are engineered in somatic cells cultured *in vitro* using the technique of gene targeting. Genetically modified cells are then used as nuclear donors to produce *inter alia*, live animals.

The technique of nuclear transfer allows the production of offspring by the reconstruction of an early embryo. Genetic material from a donor cell or karyoplast is transferred to a suitable recipient cell from which the nuclear or genomic genetic material has been removed. In the first demonstrations of this technique, successful development was only obtained when the donor genetic material was taken from blastomeres from early embryos. Subsequently, development has been obtained using donor genetic material from differentiated cells maintained in culture and isolated from embryonic (Campbell *et al.*, Nature 380, 64-66, 1996), fetal and adult tissues (Wilmut *et al.*, Nature 385, 810-813, 1997); these reports form the basis of patent applications WO 97/07669 and WO 97/07668 which are incorporated into the present application in full, including all tables and diagrams.

Methods of nuclear transfer have also been described in published patent applications WO98/39416, WO98/30683, WO98/07841, WO97/37009, WO98/27214, WO99/01163 and WO99/01164.

Live offspring have been obtained in the mouse using quiescent cell populations derived directly *ex vivo* as nuclear donors (Wakayama *et al.*, Nature 394, 369-373 1998). The successful use of differentiated cells has also been demonstrated in sheep (Wilmut *et al.*, Nature 385, 810-813, 1997), cattle (Kato *et al.*, Science 282, 2095-2098, 1998; Wells, *et al.*, Theriogenology 1, 217, 1999; Zakhartchenko, *et al.*, Theriogenology 1, 218, 1999; Vignon, *et al.*, Theriogenology 1, 216, 1999) and mice (Wakayama *et al.*, Nature 394, 369-373).

In all the above cited references the nuclear donor and the recipient cell are taken from the same species. However, there has been success reported in achieving development from embryos reconstructed using nuclear donor and recipient cells from different species (Dominko, et al., Theriogenology 49, 385, 1998; Mitalipova, *et al.,* Theriogenology 49, 389, 1998).

The use of nuclear transfer technology has many proven and potential benefits and uses in the production of mammalian embryos, fetuses and offspring. These include but are not limited to;

1. The ability to carry out genetic modification of cultured cells to be used as nuclear donors prior to embryo reconstruction.
2. The ability to carry out multiple genetic modifications in a single animal either by multiple genetic modifications of a cell population in culture or by sequential genetic modification, nuclear transfer and re-isolation of a cell population from the embryo, fetus or animal so produced.
3. The ability to increase the lifespan of cultured cell populations to be used for genetic modification by nuclear transfer and re-isolation of a cell population from the embryo, fetus or adult animal so produced.
4. The ability to produce multiple copies of an animal from a genetically modified, selected and cloned cell population.
5. The ability to produce multiple copies of any embryo, fetus or adult animal by nuclear transfer from cells taken directly *ex vivo,* or cell populations derived from any tissues taken from any of these stages with or without culture *in vitro.*
6. The ability to produce true clones (which share not only nuclear genetic identity, but also mitochondrial genetic identity) by utilising oocytes from the maternal line of the cell donor as cytoplast recipients for embryo reconstruction.
7. The ability to store intact genomes for long periods (e.g. by freezing cell populations in liquid N_2) and to use these stored cells subsequently for the production of offspring by nuclear transfer.

8. The ability to dedifferentiate somatic nuclei and to produce undifferentiated cells that may be used for production of chimeric embryos, fetuses and adult animals by embryo aggregation, or injection, or to produce embryonic stem, or embryonic germ cell populations.

9. The ability to dedifferentiate any somatic cell type by nuclear transfer and to isolate from the embryo so produced, embryonic stem cells, or any other desired specialised or unspecialised cell type e.g. neurones.

10. The possibility of achieving any of the objectives outlined in 1-9, by using nuclear donor and recipient cells from different species.

This process may be coupled with genetic manipulation techniques for the production of transgenic offspring (Schnieke *et al.*, Science 278, 2130-2133, 1997). The use of nuclear transfer coupled to genetic modification of cells in culture and their selection prior to animal production has a number of proven advantages, including;

1. Production of non-mosaic animals ensuring germ line transmission of the genetic modification/s (Schnieke *et al., Supra*).

2. An increased efficiency in the production of such genetically modified animals (Schnieke *et al., Supra*).

3. The production of multiple copies of the offspring thereby reducing the generation interval to produce flocks or herds of commercially important animals or increasing the numbers of animals for dissemination of genetic modification into the population as a whole (Cibelli, *et al.*,. Nat. Biotechnol. 16, 642-6, 1998)

The existing and published nuclear transfer technology coupled to genetic modification of cells in culture provides animals containing single and multiple genetic modifications where the transgenes are incorporated at unselected locations within the host genome. However, the production of cultured cells which incorporate a desired genetic modification engineered at a precise and predetermined location in the host genome (gene targeting) has only been described previously in the art for the

mouse using ES cells and the published methods do not involve nuclear transfer technology. No such equivalent methods exist for other mammals. Such modifications would have a number of advantages when applied to a variety of animal species including cattle, sheep, goats, horses, camels, rabbits and rodents. Such advantages include, but are not limited to:

1. The production of transgenic animals with superior transgene expression characteristics by placing the transgene at a predetermined site.
2. The removal, modification, inactivation or replacement of a chosen endogenous gene or genes and /or its control sequences.

It is an objective of this patent specification to rectify this situation and describes for the first time, methods which can be utilised to produce such modified cells which can then optionally be used to make, *inter alia* whole animals by nuclear transfer.

The present invention provides, according to a first aspect, a method of preparing a somatic cell for nuclear transfer, comprising modifying the genetic material of the somatic cell by a genetic targeting event. Preferably the somatic cell is a primary somatic cell. The somatic cell is an animal somatic cell. Preferably the cell is not an immortalised cell. Traditionally, cells can be defined as either "somatic", or "germ-line". Some cells, e.g. ES cells may not fall easily in either of these two traditional categories because they are derived from embryos before distinct somatic and germ lineages can be distinguished. Their functional equivalent, EG (Embryonic germ) cells are more easily defined as "germ-line" cells because they are derived from primordial germ cells. In the present text, the term "somatic" does not cover ES or EG cells.

The use of this method is not restricted to a particular donor cell type. Suitable cells include embryonic, fetal and adult somatic cells (of normal karyotype). In this text an "adult" cell or an "adult" animal is a cell or animal which is born. Thus an animal and

its cells are deemed "adult" from birth. Such adult animals, in fact, cover animals from birth onwards, and thus include "babies" and "juveniles". The invention includes the use of undifferentiated e.g. somatic stem cells such as haemopoietic stem cells, at least partially differentiated and fully differentiated cells. Examples of partially differentiated cells include cells of embryonic lineage or precursor cells (e.g. neural precursor cells).

Suitable somatic cells according to the first aspect of the invention are preferably, but not necessarily, in culture. Suitable somatic cells include fibroblasts, epithelial cells, endothelial cells, neural cells, keratinocytes, hematopoietic cells, melanocytes, chondrocytes, lymphocytes (B and T), avian erythrocytes, macrophages, monocytes, mononuclear cells, cardiac muscle cells, other muscle cells, granulosa cells, cumulus cells and epidermal cells. The cells for the method of the first aspect of the invention may be obtained from a variety of different organs such as skin, mesenchyme, lung, pancreas, heart, intestine, stomach, bladder, major blood vessels kidney, urethra, reproductive organs etc. or a disaggregated preparation of a whole or part of a fetus or embryo.

Suitable cells may be obtained or derived from any animal, including birds such as domestic fowl, amphibian species and fish species. In practice however, it is mammalian animals that the greatest commercial advantage is envisaged. A preferred animal from which the somatic cell is obtained is an ungulate, selected from a bovid, ovid, cervid, suid, equid or camelid. In particular, the ungulate is a cow or bull, sheep, goat, bison, water buffalo or pig. Also contemplated by the present invention are somatic cells obtained or derived from a human, horse, camel, rodent (e.g. rat, mice) or a lagomorph (e.g. rabbit).

Suitable sources for recipient cells in the nuclear transfer process are not limiting. The recipient cell is preferably an oocyte, a fertilised zygote or a two cell embryo, all of which have been enucleated.

The genetic targeting event may be any which enables modification of the genetic material of the somatic cell at a predetermined site. Such a genetic targeting event includes inactivation, removal or modification of genetic material: upregulation of the function of genetic material; replacement of genetic material; and introduction of genetic material. The genetic material in particular comprises a gene or a part thereof or a region which influences the expression of a gene or genes. Accordingly, the genetic target events may include inactivation, removal or modification of a gene, upregulation of a gene, gene replacement or transgene replacement at a predetermined locus.

Preferably the genetic targeting event occurs by homologous recombination.

The precise details of genetic targeting can be varied to improve the efficiency. In accordance with the variety of modifications, genetic targeting of a somatic cell suitable for nuclear transfer can be designed to maximise the frequency of homologous targeting. One factor which can be used to increase genetic targeting by homologous recombination is the effect of transcription on the target genetic material. Preferably, the genetic target is actively transcribed or is adjacent to a genetic locus which is actively transcribed. Such genetic targets or loci can be identified depending on the type of somatic cell used, and also possibly on the stage of such a cell. Such genes can be identified on the basis of the abundance of the corresponding mRNA molecules. Suitable genes would produce mRNAs which fall into the arbitrarily defined intermediate, or abundant class of mRNAs which are present at 300 or more copies of each molecule per cell (Alberts *et al.* 1994, Molecular Biology of the Cell, Garland Publishing, New York and London). An example of a suitable gene target in fibroblasts is any gene or locus encoding collagen, in particular the COLIA 1 or the COLIA 2 gene loci. However many suitable loci are present in a cell's genetic material.

A particularly useful strategy to increase genetic targeting is the artificial induction of gene expression or induction of chromatin changes in a somatic cell type. Preferably the genetic targeting event is facilitated by an agent which inhibits histone deacetylation or by a factor which stimulates transcription at the target locus. The factor may be expressed in the host cell. This strategy is described in more detail in the remainder of this text.

In accordance with the genetic targting of the present invention, it may be useful or necessary, at some point after the event and preferably before any nuclear transfer to remove portions of genetic material from the cell. Such material may be a selectable marker, or for example, an introduced genetic transcription activator. Such removal can be carried out by procedures described hereinafter , or by other procedures well known in the art.

The preparation of the somatic cell (by modification of the genetic material of the cell) may be a precursor step to nuclear transfer. The present invention provides, for the first time, a method by which a somatic cell can be genetically modified and which also supports successful nuclear transfer. Supporting successful nuclear transfer requires that the reconstituted embryo proceeds to produce either a live born viable animal, or an embryo or fetus which can be used as a source of tissue, including EG cells and ES cells.

The method of nuclear transfer for the present invention is not limited. Any method of nuclear transfer may be used. The nuclear transfer may include genetic material from one animal species or one animal type to a recipient cell of the same or different animal species or type.

A second aspect of the present invention provides a method of nuclear transfer, comprising a method of preparing a somatic cell for nuclear transfer (as set out in the

first aspect of the invention) and a method comprising the transfer of the genetic material from the somatic cell to a recipient cell.

As described above, all and any method of nuclear transfer may be used according to the second aspect of the invention. The somatic cell may be as described above for the first aspect of the invention. The recipient cells may be any suitable recipient cell for any method of nuclear transfer, including an oocyte, a fertilised zygote or a two cell embryo which has been enucleated.

The transfer of the genetic material from a somatic cell to a recipient cell provides an animal embryo (the animal embryo comprises the single cell result of the nuclear transfer through to any and all multi-cell stage).

The method according to the second aspect of the invention may also comprise the production of cloned totipotent or pluripotent cells from an embryo or fetus derived by nuclear transfer. Methods for the derivation of totipotent or pluripotent cells, such as stem cells, from embryos (ES cells and ES-like cells) and from primordial germ cells of later stage embryos and fetuses (EG cells) have been described by many authors as cited later in this text. Pluripotent stem cells are particularly useful for the production of differentiated cells and their precursors *in vitro* which may provide a source of cells for transplantation (preferably for humans).

Cells (including somatic cells) derived from embryos or fetuses (or adults) may also be used for further rounds of nuclear transfer. Rederivation of cells from an embryo or fetus produced by nuclear transfer may facilitate multiple or sequential genetic manipulations which are otherwise not possible in the initial primary cells.

Alternatively, the method of obtaining an animal embryo according to the second aspect of the invention may further comprise causing an animal to develop to term from the embryo. In such a method, the animal embryo is preferably developed to

term *in vivo*. If development up to blastocyst has taken place *in vitro*, then transfer into a surrogate animal takes place at this state. If blastocyst development has taken place *in vivo*, although in principal the blastocyst can be allowed to developed to term in the pre-blastocyst host, in practice the blastocyst will usually be moved from the temporary pre-blastocyst recipient and, after dissection from the protective medium, will be transferred to the permanent post blastocyst recipient. Development from the embryo to an adult goes through the stage of a fetus.

A third aspect of the invention provides a transgenic somatic cell, suitable for nuclear transfer, obtained by a method according to the first aspect of the invention.

A fourth aspect of the invention provides a transgenic embryo or fetus obtained by a method according to the second aspect of the invention.

A fifth aspect of the invention provides a method for preparing a transgenic animal comprising causing an animal to develop to term from an embryo or fetus according to the fourth aspect of the invention. Optionally, the transgenic animal of the fifth aspect may breed and such offspring (including embryos, fetuses and adults) are also encompassed by the present invention.

A sixth aspect of the invention provides a transgenic animal, or offspring thereof, according to the fifth aspect of the invention.

A seventh aspect of the invention provides a method for obtaining clonal pluripotent or totipotent cells (including a clonal pluripotent or totipotent cell population), the method comprising culturing a cell from a transgenic embryo or transgenic fetus, according to the fourth aspect of the invention, or from an adult developed from such an embryo or fetus.

An eighth aspect of the invention provides a clonal pluripotent or totipotent cell or cell population obtained by a method according to the seventh aspect of the invention.

A ninth aspect of the invention provides a method for modifying the genetic material of a somatic cell while maintaining the totipotency of the cell, the method comprising a gene targeting event.

A tenth aspect of the invention relates to the use of artificial induction of gene expression or induction of chromatin changes prior to genetic targeting event. The genetic targeting event may be in any cell, including a somatic cell, a germ line cell, and ES cell and an EG cell. This aspect of the invention includes the artificial induction of gene expression or induction of chromatin changes in a cell, to either enable or increase the frequency of genetic targeting. The genetic targeting is facilitated by the artificial induction of gene expression or by the induction of chromatin changes in a cell. Induction of chromatin changes may involve the use of agents which inhibit histone deacetylation (e.g. sodium butyrate or trichostatin A) to induce an open chromatin configuration and/or gene expression at the target locus before genetic targeting. The artificial induction of gene expression preferably involves the use of an appropriate transcriptional activator. Such an activator may be a "factor" expressed in the host cell.

Details in respect of the artificial induction of gene expression or induction of chromatin changes to either enable or increase the frequency of gene targeting in a cell are described later on in the present text. All details with respect to such genetic targeting described in this text are included in this tenth aspect of the invention in as far as it relates not only to somatic cells, but also to germ line cells, to ES and to EG cells.

The tenth aspect of the invention includes the use of artificial induction of gene expression or induction of chromatin changes in any cell type in combination with

nuclear transfer. Accordingly, the tenth aspect of the invention includes a method of preparing any cell type for nuclear transfer comprising the artificial induction of gene expression or induction of chromatin changes to facilitate a genetic targeting event. Such a method may optionally also include transfer of genetic material from the cell into a suitable recipient cell (ie a nuclear transfer step). The method may be used to produce clonal totipotent or pluripotent cells and/or a transgenic embryo or animal. The totipotent or pluripotent cells may be obtained directly from cells after nuclear transfer or from the produced embryo or animal.

For all aspects of the invention which include a gene targeting event, it is clear that more than one gene targeting event may take place. For example, in a single primary cell there may be a gene targeting event to remove or inactivate genetic material and a gene targeting event to introduce a transgene. More than two gene targeting events are also envisaged. The more than one gene targeting event may take place simultaneously, subsequently or sequentially in the same passage or a different passage. Alternatively a second or further gene targeting event may take place in a cell derived from an embryo, fetus or adult which was developed from an original somatic cell according to the present invention.

The process of embryo reconstruction and production of viable offspring by nuclear transfer is a multistep procedure, each of these will now be described in detail. Also described are more details with respect to gene targeting according to the present invention. All details which follow apply to the invention herein described. In addition, the following terms are referred to in this text and their full expressions are set out here:

ES cell - embryonic stem cell;
EG cell - embryonic germ cell;
MII - metaphase II;
PCR polymerase chain reaction;

HR - homologous recombination;

AAV - adeno associated virus;

DAF - decay accelerating factor;

MCP - membrane cofactor protein;

CD59 - membrane inhibitor of reactive lysis;

HPRT - hypoxanthine phosphoribosyltransferase;

gpt - xanthine guanine phosphoribosyltransferase;

LIF - Leukaemia inhibitory factor;

GFP - green fluorescent protein;

IRES -internal ribosomal entry site

The Recipient Cell or Cytoplast.

Oocytes, fertilised zygotes and two cell embryos have been used as cytoplast recipients for nuclear transfer. In general, oocytes arrested at metaphase of the second meiotic division (also termed unfertilised eggs, or MII oocytes) have become the cytoplast of choice. At this point in oocyte development the genetic material is arranged upon the meiotic spindle and is easily removed using mechanical means. Several reports have demonstrated that during maturation i.e. between the germinal vesicle stage (prophase of the first meiotic division) and arrest at metaphase of the second meiotic division genomic DNA can be removed and the resulting cytoplast used for nuclear transfer (Kato *et al., Mol Reprod Dev* 36, 276-8, 1993). The use of fertilised zygotes as cytoplast recipients has been reported in mouse (Kwon *et al.,* Proc Natl Acad Sci U S A, 93, 13010-3, 1996), cattle (Prather *et al.,* J. Reprod Fertil Suppl 41, 1990), and pigs (Prather *et al.,* Biol.Reprod. 41, 414-8, 1989). In cattle and pigs, development of embryos reconstructed using zygotes as cytoplast recipients is low and on the whole restricted to the exchange of pronuclei suggesting that factors essential for successful development are removed with the pronuclei.

Preparation of a Cytoplast Recipient by Removal of the Genomic Genetic Material.

This process has in general been termed enucleation. In the majority of recipients utilised, the genomic DNA is not enclosed within a nuclear membrane at the time of removal. The removal of the genetic material according to the present invention and described by the term "enucleation" does not require that the genetic material is present in a nuclear membrane (it may or may not be, or may partially be). Enucleation may be achieved physically by actual removal of the nucleus, pronuclei or metaphase plate (depending on the recipient cell), or functionally, such as by the application of ultra-violet radiation or other enucleating influence. Removal of the genetic material is possible by physical and or chemical means. In the early reports of nuclear transfer, MII oocytes were simply cut in half on the basis that one half would contain the genetic material and the other would not. Modifications to this approach have been made in order to reduce the volume of cytoplasm, which was removed. This may be achieved by aspiration of a small amount of cytoplasm from directly beneath the 1st polar body using glass micropipettes or by using a knife to cut away that part of the oocyte beneath the polar body. To facilitate plasticity of the oocyte it may be pre-treated with cytochalasin B or other such agent that disrupts the cytoskeleton. In contrast to physical enucleation, chemical treatment has been demonstrated to cause complete removal of the genetic material in the mouse. Treatment of maturing oocytes with the topoisomerase inhibitor ectoposide results in the expulsion of all genomic material with the 1st polar body (Elsheikh *et al.*, Jpn J Vet Res 45, 217-20, 1998), however no development to term has been described using cytoplast recipients produced by this method and there are no reports of this procedure in other species. Centrifugation of MII oocytes combined with Cytochalasin B treatment has been reported to cause enucleation in hamster and cattle oocytes (Tatham *et al.*, Hum Reprod 11, 1499-503, 1996). The development of embryos reconstructed from such cytoplasts has been reported in cattle however the frequency of development is low.

When using zygotes the genetic material may be removed by mechanical aspiration of both pronuclei. Dependent upon species, in order to facilitate visualisation of the pronuclei the zygotes may be centrifuged prior to enucleation.

Introduction of genetic material (embryo reconstruction).

Having prepared a suitable recipient cell or cytoplast the donor genetic material must be introduced. Various techniques have been reported including;

1. Cell fusion induced by chemical, viral or electrical means.
2. Injection of an intact cell by any method
3. Injection of a lysed or damaged cell.
4. Injection of a nucleus.

Any of these methods may be used in any species or in a combination of species with some modifications of individual protocols.

Activation of the reconstructed embryo.

In addition to the transfer of donor genetic material from the karyoplast to the cytoplast, the cytoplast must be stimulated to initiate development. When using a fertilised zygote as a cytoplast recipient, development has already been initiated by sperm entry at fertilisation. When using MII oocytes as cytoplast recipients the oocyte must be activated by other stimuli. Various treatments have been reported to induce oocyte activation and promote early embryonic development including but not limited to; application of a DC electric stimulus, treatment with ethanol, ionomycin, inositol tris-phosphate (IP_3), calcium ionophore A23187, treatment with extracts of sperm or any other treatment which induces calcium entry into the oocyte or release of internal calcium stores and results in initiation of development. In addition any of these treatments in combination, their application at the same or different times or in combination with inhibitors of protein synthesis (i.e. cycloheximide or puromycin) or inhibitors of serine threonine protein kinases (i.e. 6-DMAP) may be applied.

Culture of reconstructed embryos.

Nuclear transfer reconstructed embryos may be cultured *in vitro* to a stage suitable for transfer to a final recipient using any suitable culture medium or culture process. The reconstituted embryos may otherwise be cultured *in vitro* until they are used in the production of cloned totipotent or pluripotent cells, according to the second aspect of the invention. Alternatively, embryos may be cultured *in vivo* in the ligated oviduct of a suitable host animal (in general, sheep) until a stage suitable for transfer to a final surrogate recipient is reached in order for the animal to be grown to term. Embryos from cattle, sheep and other species may be cultured in a trans species recipient; for simplicity a sheep provides a suitable recipient for bovine, ovine and porcine species. It is usual to embed the embryos in a protective layer of agar or similar material to prevent mechanical damage to the reconstructed embryos or attack by macrophages whilst in the oviduct of the temporary recipient.

Gene targeting and embryonic stem cells

Gene targeting by homologous recombination between an exogenous DNA construct and cognate chromosomal sequences allows precise modifications to be made at predetermined sites in the genome. Gene targeting is well established in mouse embryonic stem (ES) cells, and has been used to effect modifications in a large number of murine genes (summarised by Brandon *et al.,* Curr. Biol. 5, 625-634, 758-765, 873-881, 1995). This has been facilitated by the ease with which genetic modifications engineered in ES cells *in vitro* can be transferred to whole mice and the consequences of gene targeting studied (reviewed by Papaioannou and Johnson, In: Gene targeting: a practical approach. Ed Joyner, A.L. Oxford University Press, 1992; and by Ramirez-Solis and Bradley Curr Opin. Biotech. 5, 528-533, 1994). Mouse ES cells are pluripotent cells derived from early embryos (Evans *et al.,* Nature 292, 154-156. 1981) which can be grown and manipulated *in vitro* then reintroduced into the preimplantation embryo where they can contribute to all cell types of a chimeric animal, including germ cells (Robertson, E.J., (Ed.) 1987. Teratocarcinomas and embryonic stem cells. A practical approach. IRL Press, Oxford.).

The potential benefits of engineering precise genetic modifications by gene targeting in species other than mice, e.g. livestock, have been described many times (e.g. Colman and Garner, Pharmaceutical Forum 5, 4-7, 1996). These include, but are not limited to, the production of human therapeutic proteins in the body fluids, disease prevention, increasing required production traits, cell based therapies, cell based delivery systems for genetic therapy, tissue and organ transplantation.

Great efforts have therefore been made to derive ES lines from a wide variety of species. However, definitive ES cell lines i.e. capable of contributing to the germ line of a chimeric animal, have not been demonstrated from any mammalian species other than mouse. The status of human ES cells (Thomson *et al.,* Science 282, 1145-7, 1998), remains unknown in this respect because of ethical constraints. There are reports of ES or rather ES-like cell lines derived from hamster, mink, sheep (Piedrahita *et al.,* Theriogenology 34, 879-901, 1990), cattle (Stice *et al.,* Biol. Reprod. 54, 100-110, 1996), pig (Gerfen *et al.,* Anim. Biotechnol. 6, 1-14, 1995) and rhesus monkey (Thomson *et al.,* Proc. Natl. Acad. Sci. USA 92, 7844-7848, 1995). In all of these cases, the more limited definition of "cells which under the appropriate *in vitro* conditions, can differentiate along at least three different lineages" has been used to support claims that the cells derived represent ES cells. The production of pig (Wheeler, Reprod. Fertil. Dev. 6, 1-6, 1994) and rat chimeras have also been reported, although in neither case has ES contribution to the germ line been demonstrated.

Embryonic germ (EG) cells are undifferentiated cells functionally equivalent to ES cells, that is they can be cultured and transfected *in vitro* then contribute to somatic and germ cell lineages of a chimera (Stewart, Dev. Biol. 161, 626-628, 1994). EG cells are derived by culture of primordial germ cells, the progenitors of the gametes, with a combination of growth factors: leukaemia inhibitory factor, steel factor and basic fibroblast growth factor (Matsui *et al.,* Cell 70, 841-847,1992; Resnick *et al.,* Nature 359, 550-551, 1992).

Several attempts have been made to isolate EG lines from primordial germ cells in cattle (Cherny, *et al.,* Theriogenology 41, 175, 1994; Stokes, *et al.,* Theriogenology 41, 303, 1994), pig (Shim, *et al.,* Biol. Reprod. 57, 1089-1095, 1997; Piedrahita *et al.,* Biol. Reprod. 58, 1321-1329,1998) and rat (Mitani, *et al.*, Theriogenology 41, 258, 1994). Blastocyst injection of cultured EG cells led to production of mid-gestation chimeric bovine embryos (Stokes, Theriogenology 41, 303, 1994). More recently chimeric male piglets have been produced from both genetically manipulated (Piedrahita, *et al.,* Biol. Reprod. 58, 1321-1329,1998) and normal EG cells (Shim, *et al.,* Biol. Reprod. 57, 1089-1095, 1997). In both instances EG cell contribution to the testes was detected. Unfortunately the ability of this approach to achieve germline transmission could not be established, as one of the animals was stillborn and the other failed to thrive and was sacrificed.

The lack of fully functional large animal ES, or EG cells has however been circumvented by developments in nuclear transfer which allow genetic modifications to be made to somatic cells in culture and then those cells used as nuclear donors to produce a whole animal, as demonstrated by Schnieke *et al.* (*Supra*).

Gene targeting in somatic cells

There has been considerably less work on gene targeting in somatic cells.

The efficiency with which gene targeted clones can be derived is a function of the frequency of homologous recombination (HR) events and the frequency of random integration events. In all mammalian cell types, HR events are significantly less frequent than random events. Distinguishing HR events against a background of random integrants represents a major obstacle to the isolation of gene targeted clones. The results of published experiments indicates that gene targeting in somatic cells is infrequent, estimates of the ratio of HR to random events range from 1:50 or 1:230 in fibrosarcoma (Itzhaki *et al.,* Nat. Genet. 15, 258-265,1997), 1:241 in myeloid leukemia cells (Zhen *et al.,* Proc. Natl. Acad. Sci. USA 90, 9832-9836, 1993), 1:1000

in bladder carcinoma (Smithies *et al.*, Nature 317, 230-234, 1985), to 1:9700 in erythroleukemia /lymphoblast fusion cells (Shesely *et al.*, Proc. Natl. Acad. Sci. USA 88, 4294-4298, 1991). However, these data were obtained from a range of immortalised cell lines and describe the targeting of different gene loci with different gene targeting constructs.

In those few cases where the same construct has been used to target the same locus in both ES cells and somatic cells, the comparative data indicates that the ratio of HR to random integration events may be significantly lower in somatic cells (Arbones *et al.*, Nat. Genet. 6, 90-97, 1994). Recombinogenic cells of the immune system, such as B cells, represent a special exception (Buerstedde *et al.*, Cell 67, 179-188, 1991; Takata, M. *et al.*, EMBO J. 13, 1341-1349, 1994).

Comparison of the frequency of homologous recombination between immortalised and primary somatic cells indicates that homologous recombination is less frequent in primary cells than in immortalised cell lines (Finn *et al.*, Mol. Cell. Biol. 9, 4009-4017, 1989; Thyagarajan *et al.*, Nuc. Acids Res. 24, 4084-4091, 1996). This is important to the production of animals by nuclear transfer because normal, euploid, non-immortalised cells are preferred as nuclear donors because of the risk that immortalised cells may not support development or may lead to tumours in the resultant animal.

Factors affecting the frequency of gene targeting.

Numerous factors have been identified as affecting the frequency of gene targeting. As stated above, there are less data available regarding factors critical to targeting in somatic cells than ES cells.

The frequency of gene targeting in somatic cells has been shown to increase dramatically with the length of the region of homology in the targeting vector (Scheerer, *et al.*, Mol. Cell. Biol. 14, 6663-6673, 1994).

The use of DNA isogenic to the host cell has been demonstrated to improve the frequency of gene targeting in embryonic stem cells (Deng *et al.,* Mol Cell Biol. 12, 3365- 3371, 1992; te Riele *et al.,* Proc. Natl. Acad Sci. USA 89, 5128-5132, 1992). However this has not been investigated in somatic cells.

The effect of transcription of the target gene on the frequency of homologous recombination is the subject of some dispute. Some of the earliest reports of gene targeting demonstrated targeting modifications made to genes which are inactive in the cell type used (Smithies *et al.,* Nature, 317, 230-234, 1985; Johnson, *et al.,* Science 245, 1234- 1236, 1989). However, it has been proposed that homologous recombination at a gene locus is more frequent when that gene is actively transcribed than when it is inactive (Nickolof *et al.,* Mol. Cell. Biol. 10, 4837-4845, 1990; Thyagarajan *et al.,* Nucleic Acids Res. 23, 2784-2790, 1995). This proposal has been disputed by Yanez and Porter, who report no correlation between gene targeting frequency and the transcriptional status of the interferon inducible 6-16 gene in human HT1080 cells (Gene Therapy, 5, 149-159, 1998).

The presence of double strand breaks at the target locus has been shown to stimulate gene targeting in CHO cells (Liang *et al.,* Proc. Natl. Acad Sci. USA 93, 8929-8933, 1996) but not mouse Ltk$^-$ cells (Lukacsovich *et al.,* Nuc. Acids Res. 22, 5649-5657, 1994). However double strand breaks have also been shown to stimulate the level of illegitimate recombination in CHO cells (Sargent *et al.,* Mol Cell Biol. 17. 267-277, 1997). The consequent risk of introducing genetic aberrations reduces the usefulness of this approach for cells destined for nuclear transfer. This also applies to other methods of stimulating homologous recombination by induction of DNA damage, e.g. chemical carcinogens, UV radiation, gamma radiation and photoreactive molecules (reviewed by Yanez and Porter, Gene Therapy, 5, 149-159, 1998).

Yanez and Porter (op. cit) also review other factors which affect the rate of homologous recombination. These include the method of transfection, the growth conditions of the host cell culture, the stage of the cell cycle of the host cell, modification of the ends of transfected DNA, the inactivation of the MSH2 gene and the inhibition of poly ADP ribose polymerase enzyme activity.

Methods of enriching or identifying gene targeting events

There are several methods whereby cells carrying possibly rare targeting events can be enriched, or identified from a transfected population.

Transfectants can be divided into pools and the presence of targeted clones within each pool screened, typically by the polymerase chain reaction. Positive pools are then progressively subdivided and rescreened until a single clone has been isolated (Shesely, *et al.*, Proc. Natl. Acad. Sci. USA 88, 4294-4298, 1991). However, such schemes are not ideally suited to primary cells destined for nuclear transfer, because repeated rounds of purification extend the time in culture. Extended culture is undesirable because primary cells may become senescent or acquire genetic aberrations.

The use of gene targeting vectors which maximise the target frequency and allow selection of targeted clones without increasing the time spent in culture is more appropriate for cells destined for nuclear transfer. Gene targeting strategies such as the enhancer trap, promoter trap and polyadenylation trap are described in detail by Hasty and Bradley (In: Gene targeting: a practical approach. Ed Joyner, A.L. Oxford University Press, 1992) and are discussed later in this text.

Other gene targeting methods

Very high rates of homologous recombination have been reported using chimeric RNA/DNA oligonucleotides for targeting the β-globin gene in lymphoblastoid cells

(Cole-Strauss, Science 273, 1386-1389, 1996) and the Factor IX gene both *in vitro* and *in vivo* (Kren, *et al.*, Nature Med. 4, 285-290, 1998). Unfortunately the technology does not seem to transfer easily and other researchers have failed to apply it to their gene of choice (Strauss, Nature Medicine 4, 274-275, 1998).

High targeting frequencies have also been observed with gene targeting vectors based on the adeno-associated virus (AAV). Russell *et al.* (Nature Genetics 18, 325-330, 1998) report 11 out of 13 transfected primary human fibroblasts had a correctly targeted hypoxanthine phosphoribosyltransferase gene.

Both of these methods are limited in the type of modification that can be engineered, because only a few nucleotide changes can be made at the target locus. While this allows gene inactivation e.g. by the insertion of stop codons, or subtle modification by the substitution of individual amino acids, these methods do not allow the insertion or replacement of larger regions which are necessary for gene replacement or transgene placement.

In this text, there is described the generation, identification and isolation of somatic cells carrying predetermined genetic modifications at a defined locus, "gene targeting" while maintaining the ability of those cells to support production of, following nuclear transfer, either i) a fetus or ii) a viable animal or iii) pluripotent cell populations.

While the method is applicable to all mammalian species, the preferred species are sheep, cattle (cow and bull), goat, pig, horse, camel, rabbit, rodent and human. This invention does not relate to human reproductive cloning. It does cover human tissue cells and, where applicable, human embryos, in particular, embryos under 14 days old.

Preferably, gene targeting is carried out at, or adjacent to, a gene locus which is actively transcribed, or otherwise capable of supporting gene targeting at high frequency. Many somatic cell types are capable of supporting nuclear transfer and are

thus suitable according to the present invention (e.g. mammary epithelial cells, fetal fibroblasts, adult fibroblasts, oviduct epithelial cells, granulosa cells and cumulus cells). Many other cell types will also support nuclear transfer, including embryonic stem cells and their differentiated derivatives, endothelial cells and sub-endothelial cells. Gene targeting of somatic cells in combination with nuclear transfer allows more flexibility than gene targeting using ES cells alone. ES cells are a single cell type which display a single pattern of gene expression. The wide choice of somatic cells available for nuclear transfer allows a cell type to be chosen for gene targeting in which the gene locus of interest is preferably transcriptionally active, or capable of supporting gene targeting at high frequency. Thus, the frequency of homologous recombination can be maximised.

This strategy can be extended to include the artificial induction of gene expression or induction of chromatin changes in a somatic cell type to either enable or increase the frequency of gene targeting. Induction of chromatin changes may involve the use of agents which inhibit histone deacetylation (e.g. sodium butyrate or trichostatin A) to induce an open chromatin configuration and/or gene expression at the target locus before gene targeting.

Gene expression and histone acetylation

There is strong evidence that gene activity is correlated with the acetylation of core histones (reviewed by Jeppeson, Bioessays, 19, 67-74, 1997; Pazin and Kadonaga, Cell 89, 325-328, 1997) and that histone deacetylation regulates gene transciptional activity (Wolffe, Science 272, 371-372, 1996). Detailed mechanisms of repression involving proteins with histone deacetylase activity have been elucidated for several genes (e.g. Brehm *et al.*, Nature 391, 597-601, 1998; Magnagi-Jaulin *et al.*, Nature 391, 601-604, 1998; lavarone *et al.*, Mol Cell Biol 19, 916-922, 1999). The inhibition of histone deacetylase activity by chemical reagents such as sodium butyrate, or trichostatin A has been shown to reactivate silent genes (Chen *et al.*, Proc. Natl. Acad. Sci. USA 94, 5798-5803, 1997) and inhibit transcriptional repression (Brehm *et al.*,

Nature 391, 597-601, 1998; Magnagi-Jaulin *et al.*, Nature 391, 601-604, 1998). The effect of inhibitors of histone deacetylase on the frequency of gene targeting has not been described in the art.

Alternatively, particular genes or classes of genes can be activated using particular transcriptional activators. Such transcriptional activators may be termed "factors". For example, Weintraub *et al* (Proc. Nat. Acad. Sci. USA, 86, 5434-5438, 1989) show that primary human and rat fibroblasts, when transfected with the transcriptional activator, MyoD, turn on a variety of muscle genes, and in the case of the rat cells, can form myotubes. Likewise, Tontonoz *et al* (Cell, 79, 1147-1156, 1994) have shown that the regulators PPARg and C/EBPa synergise to powerfully promote adipogenesis in fibroblasts. In the context of the protocols described in this text, targeting to genes which are normally active in muscle or fat cells can be effected by the introduction of the type of factors described above. However, it is important that the method used to introduce a transcription factor does not compromise the ability of the cells to support nuclear transfer.

Expression of transcription factors can be achieved by transient transfection of an expression construct prior to gene targeting. Because only a small proportion of cells transiently transfected actually integrate the exogenous DNA into their genome, a transcription activator designed to effect activation of a gene at the target locus can be expressed prior to gene targeting and will in the majority of cells leave no integrated DNA. The presence or absence of integrated transcription activator construct DNA can be determined in individual clones and those which lack integrated copies used for nuclear transfer. Where necessary, integrated copies of transcription activator expression constructs can be removed if they are flanked by recognition sites for site specific recombinase enzymes e.g. the *lox*P sites for Cre or the FRT sites for FLP recombinase (Kilby *et al.*, Trends in Genetics, 9, 413-421, 1993).

The choice of target locus may be made on the basis of the ability of that locus to support gene targeting at high frequency, or on the basis of the function of the gene. Gene targeting at the target locus may be carried out so that the expression of the target gene is reduced or ablated, increased, or left unaffected. Gene targeting does not necessarily have to affect the function of the target gene, indeed in some circumstances it may be preferable that expression and function of the target locus is left unaffected.

Gene targeting of primary somatic cells is carried out to achieve gene inactivation, gene upregulation, gene modification, gene replacement, or transgene placement at the target locus. Examples of preferred target loci and modifications include, but are not limited to:

1. inactivation, removal or modification
- of genes responsible for the presence of antigens which are xenoreactive to humans (e.g. α-1,3 galactosyltransferase),
- of the PrP locus responsible for the production of the prion protein and its normal counterpart in non human animals,
- of genes which in humans are responsible for genetic disease and which in modified, inactivated or deleted form could provide a model of that disease in animals, e.g. the cystic fibrosis transmembrane conductance regulator gene.
- of regulatory regions or genes to alter the expression of one or more genes, e.g. RFX transactivator genes which are responsible for regulation of major histocompatibility class II molecules
- of endogenous viral sequences
- of genes responsible for substances which provoke food intolerance or allergy.
- of genes responsible for the presence of particular carbohydrate residues on glycoproteins, e.g. the cytidine monophospho-N- actetyl neuraminic acid hydroxylase gene in non-human animals

2. upregulation

- of genes responsible for suppression of complement mediated lysis (e.g. porcine CD59, DAF, MCP)

- of gene expression by the introduction of a response element to allow experimental modulation of gene expression

3. gene replacement

- replacement of genes responsible for production of blood constituents (e.g. serum albumin) with their human counterpart.

- replacement of genes responsible for substances which provoke food intolerance or allergy with a more benign (e.g. human) counterpart

- replacement of immunoglobulin genes with their human counterpart.

- replacement of genes responsible for surface antigens with their human counterpart.

4. transgene placement at a predetermined locus:

- placement of a transgene at a gene locus which may offer advantageous transgene expression

- placement of a transgene at a site which places it under the control of an endogenous regulatory region.

The gene targeting vector and the experimental procedure is designed so that the time taken to identify, isolate, analyse and expand primary cell clones carrying targeted events is minimised. This is an important aspect of the invention because reduction of the time in culture increases the likelihood that cells used as nuclear donors are viable, normal and euploid. Recognised risks associated with *in vitro* culture of primary cells which are detrimental to the outcome of nuclear transfer include: senescence due to limited lifespan, acquisition of genetic damage, loss of a normal complement of chromosomes and rapid erosion of chromosomal telomeres.

Precautions which can optionally be taken to minimise the time in culture are:

1. Cryopreservation of cell samples destined for use as nuclear donors at the earliest possible stage.

2. The use of a gene targeting vector which allows direct selection or identification of homologous recombinants relative to random integrants.

3. The use of culture conditions designed to reduce the metabolic insults suffered by the primary cells. For example, by the use of a reduced oxygen atmosphere, or the inclusion of antioxidants to minimise the extent of oxidative damage. However, the invention is not limited to the use of such conditions.

The use of promoter trap, or polyadenylation trap strategies (reviewed by Hasty and Bradley In: Gene targeting: a practical approach. Ed Joyner, A.L. IRL press Oxford, 1992) are preferred embodiments of the invention. In each case, the gene targeting vector is designed such that homologous recombination between the gene targeting vector and the target locus renders a marker gene active, while in the majority of random integrants it is inactive. Marker genes may include genes which confer resistance to drugs (e.g. neomycin, G418, hygromycin, zeocin, blasticidin, histidinol) or other selectable markers (e.g. HPRT, gpt), visible markers (e.g. GFP) or other selection systems (e.g. single chain antibody /hapten systems; Griffiths *et al.*, Nature 312, 271-275, 1984). Conversely, strategies may be designed such that homologous recombination results in removal of a negatively selectable marker gene. For example a vector could be designed such that in correctly targeted cells a site specific recombinase gene e.g. the Cre or FLP recombinase (Kilby *et al.*, Trends in Genetics, 9, 413-421, 1993) is activated e.g. by a promoter or polyadenylation trap, and the recombinase results in the deletion of a toxin gene flanked by appropriate recognition sites (e.g. *loxP* or FRT sites)

The gene targeting vector is designed to maximise the frequency of homologous targeting relative to random integration events. This is achieved by incorporating large regions of DNA homologous to the target locus into the gene targeting vector. While it is desirable to maximise the size of these homologous regions, in practice these are

limited by the constraints of construct production and the requirement for a reliable, simple genetic screen (e.g. by PCR amplification) to identify targeted events. Homologous DNA in the gene targeting vector may, or may not be isogenic to the host cell, in that it may or may not be derived from the host cell, or from other cells of the same individual.

Gene targeting can be carried out such that a transgene is cointegrated with a selectable marker gene at the target locus. This could be used to provide a suitable site for achieving high expression of the transgene. A preferred embodiment of the invention is the placement of a transgene designed to express a foreign protein in the milk of a transgenic animal at a locus known, or predicted to support abundant expression in the mammary gland. Where the presence of the marker gene is undesirable in the final animal, it may be removed by the action of specific recombination systems e.g. the Cre/*lox*P system or the FLP/FRT system (Kilby *et al., Supra*) if it is flanked by recombination recognition sites. Removal of the marker gene may either be achieved in the cells before nuclear transfer, or in cells derived from animals (including fetuses) produced by nuclear transfer, or in oocytes, zygotes, or embryos which carry the genetic modification and are from a lineage which started with an animal made by nuclear transfer or during subsequent mating of animals derived by nuclear transfer. A method whereby Cre recombinase can be specifically activated and used to effect recombination during the production of male gametes has been described by O'Gorman *et al.*, (Proc. Natl. Acad. Sci. USA. 94, 14602-14607, 1997).

Multiple rounds of gene targeting may be carried out, either in cells before nuclear transfer or in cells derived from an animal produced by nuclear transfer. Retargeting of a locus may be facilitated by the inclusion of a marker gene in the first round of targeting such that its loss on subsequent targeting allows ready identification or selection of clones carrying subsequent targeted events.

The following drawings are referred to in the present text:

Figure Legends

Figure 1

Structure of the COLT-1 gene targeting vector and the targted COLIA1 locus

The upper portion shows a diagram of the COLT-1 vector with the 5' and 3' regions homologous to the ovine COLIA1 locus, the IRES neo region and the bacterial plasmid indicated.

A double crossover event at the ovine COLIA1 locus (middle) results in the structure of the targeted locus as indicated in the lower portion of the figure. Although it is not possible to determine the precise sites where recombination between the COLT-1 vector and the target locus recombination occur, regions at the target locus homologous to the COLT-1 vector are shown in darker shading.

Figure 2

PCR screening strategy used to identify gene targeting events at the ovine COLIA1 locus.

The upper portion shows the approximate position of the two primers used to amplify a 3.4 fragment from a region unique to the COLT-1 vector to a region unque to the COLIA1 locus.

Sequence A shows a portion of DNA sequence spanning the junction between the region unique to the COLIA1 locus and the 5' region of shared homology with the COLT-1 vector. The position and orientation of the COLTPCR4 primer is shown.

Sequence B shows a portion of DNA sequence spanning the junction between the IRES neo gene and the 5' region of shared homology. The position and orientation of the COLTPCR8 primer is shown.

Figure 3

Agarose gel electrophoresis of PCR fragments amplified from PDFF-2 G418 resistant cell clones. Lanes containing samples from cell clones 1, 2, 6, 12, 13, 14, and 26 are indicated. The position of the diagnostic 3.4kb amplified fragment is indicated by the arrow.

Figure 4

Sequence analysis of the 5' junction of the targetd locus in three independently derived targeted cell clones.

The upper portion shows the sequence of the 5' end of the linearised COLT-1 gene targeting vector. Terminal sequence derived from a cloning vector is indicated

The middle portion shows a portion of the sequence of the diagnostic 3.4 kb fragment amplified from each of the targeted cell clones 6, 13 and 14 spanning the junction between the region unique to the COLIA1 locus and the 5' region of shared homology with the COLT-1 vector.

The lower portion shows the sequence of the PDFF2 COLIA1 locus over the same region.

We have exemplified this approach by demonstrating gene targeting at the COLIA1 locus by placement of a selectable marker gene alone and in combination with a transgene. The COLIA1 gene expresses the alpha-1(I) procollagen gene at high level in the fibroblast cells used for targeting. The frequency of gene targeting obtained with the marker gene was slightly greater than 1 targeted event per 10 drug resistant cell

clones. This is significantly in excess of the rate of gene targeting that could be expected in primary cells based on published data. Furthermore, the cells carrying the targeted modification retain their ability to support nuclear transfer. This therefore facilitates the practical use of somatic cells coupled with nuclear transfer as a means of transferring precise predetermined genetic modifications wrought *in vitro* to whole animals.

The present invention is exemplified by the following, non-limiting examples.

Example 1

Placement of a neo marker gene at the COLIA1 locus in primary ovine fetal fibroblast cells by gene targeting

The Poll Dorset primary fetal fibroblast primary culture PDFF2 has been described previously (Schnieke *et al., Supra*). PDFF2 genomic DNA was used to generate a library of cloned DNA fragments from which a molecular clone corresponding to a portion of the ovine COLIA1 gene from exon 44 to approximately 14 kb 3' of the translational stop site was isolated. The ovine COLIA1 molecular clone was used as a source of DNA for construction of the COLT-1 gene targeting vector.

COLT-1 is designed to place the neo selectable marker gene downstream of the COLIA1 gene without disrupting the expression of the COLIA1 gene.

Gene targeting with the COLT-1 vector was designed to maximise the frequency of homologous recombination in PDFF2 fetal fibroblasts:

a. The choice of target locus, COLIA1 gene locus is highly expressed in fibroblasts.

b. The COLT-1 construct was designed as a promoter trap such that the construct alone does not confer G418 resistance. Homologous recombination at the COLIA1 locus introduces a neo gene lacking a promoter but which has at the 5' end an internal ribosomal entry site (IRES) to facilitate translation. Homologous recombination results

in insertion of the IRES neo gene into the COLIA1 transcribed region downstream of the COLIA1 translational stop site. Transcription of the targeted COLIA1 locus produces a bicistronic message with translation of the neo coding region occurring by initiation at the internal ribosomal entry site.

c. The length of the regions within COLT-1 homologous to the COLIA1 gene was maximised.

COLT-1 consists of:

A 3 kb region of the 3' end of the ovine COLIA1 gene, from a position approximately 2.9kb 5' of the translational stop site to an SspI site 131 bases 3' of the stop site.

A 0.6 kb internal ribosomal entry site (IRES) region corresponding to bases 1247 to 1856 of the IRES hygro vector (Clontech, Genbank accession number: U89672)

A 1.7 bp region containing the bacterial neo gene and a portion of the 3' end of the human growth hormone gene containing the polyadenylation site essentially the same as that described by McWhir *et al.*,(Nature Genetics 14, 223-226, 1996)

A 8.3 kb region of the 3' end and flanking region of ovine COLIA1 gene, from a SspI site 131 bases 3' of the translational stop site to a XhoI site approximately 8.4kb 3' of the stop site.

The bacterial cloning vector pSL1180 (Pharmacia)

The structure of the COLT-1 Vector and the targeted COLIA 1 locus are shown in Figure 1.

COLT-1 DNA linearised with the restriction enzyme SalI was transfected into early passage PDFF2 cells and G418 resistant cell clones were isolated as summarised below:

Day 0: $5x10^5$ cells PDFF2 cells at passage 3 were transfected with 6μg linearised COLT-1 DNA using lipofectAMINE following the procedure recommended by the manufacturers (Gibco, BRL Life Technologies).

Day 2: Transfected cells were split into two sets of eight (10cm) dishes and G418 added to the medium at either 0.8 mg/ml, or 3.0 mg/ml.

Day 11-18: Colonies were isolated and replated into 6 well dishes. Samples were taken for PCR analysis.

Day 15- 19 Colonies were expanded into 25cm^2 flasks. Further samples were taken for PCR analysis

Day 20-25 Expanded clones were cryopreserved.

Throughout, PDFF-2 cells were grown in BHK 21 (Glasgow MEM) medium supplemented with L-glutamine (2mM), sodium pyruvate (1mM) and 10% fetal calf serum in standard tissue culture flasks and dishes in a humidified tissue culture incubator using an atmosphere composed of 2% O_2, 5% CO_2, 93% N_2. Cells were passaged by standard trypsinisation.

PCR analysis was used to distinguish between random integrated copies of COLT-1 and homologous recombinants at the COLIA1 locus. The PCR scheme used is shown in Figure 2. The upper part of Figure 2 shows the predicted structure of the targeted locus and the positions of the two primers used to amplify a fragment of 3.4kb if the IRES neo cassette is inserted at the correct position in the COLIA1 gene. The DNA sequences A and B, in the lower part of the figure, show the precise positions of each primer relative to the structure of the targeted locus.

Samples of cells to be screened were lysed in PCR lysis buffer (50mM KCl, 1.5 mM $MgCl_2$, 10mM Tris pH8.5, 0.5% NP40, 0.5% Tween) plus proteinase K, and incubated at 65°C for 30min. Proteinase K was inactivated at 95°C for 10 min and polymerase chain reaction carried out using the "Expand long template PCR system" (Boehringer)

following the manufacturers recommended conditions. Thermal cycling conditions were as below:

	94°C	2 min
10 cycles of	55°C	30 sec
	68°C	2 min
	94°C	10 sec
20 cycles of	60°C	30 sec
	68°C	2 min + 20 sec/cycle
	68°C	7 min

Figure 3 shows a representative agarose gel electropherogram of PCR products amplified from seven G418 resistant cell clones. Four of these (clones 6, 13, 14, 26) show the presence of the diagnostic 3.4 kb fragment indicative of integration of the IRES neo gene into the COLIA1 locus by homologous recombination.

In total 63 clones were analysed by PCR, 7 of which (11%) were positive. The nucleotide sequence of fragments amplified from three clones (6, 13, 14) was determined and compared with the sequence of the ovine COLIA1 gene. Portions of the sequence of the fragment amplified from each clone is shown in Figure 4. It can be seen that in each case the sequence of the fragment amplified from each clone is identical over the 5' junction of the targeted locus and is consistent with a homologous recombination event occurring between the COLT-1 plasmid and the endogenous COLIA1 gene. That is, the PCR fragment contains sequence from the COLIA1 gene 5' of that present within the COLT-1 construct and does not contain the sequence known to be present at the extreme 5' end of the linearised COLT-1 construct.

Example 2. Generation of COLIA1 gene targeted sheep

Targeted clones 6 and 13 were selected for nuclear transfer primarily because they grew most readily in culture. The chromosome number of these clones was determined

as a basic requirement for competence as nuclear donors. In each case the gross chromosome number indicated that each clone was euploid.

Table 2 shows the chromosome number of clones 6 and 13

Clone	number spreads counted	number spreads 53/54[1]
6	28	17
13	42	28

1 Number of spreads with a chromosome count of either 53 or 54.

It was calculated that the total period of culture these cells had undergone since disaggregation of the original fetus up to the point of preparing multiple cryopreserved stocks ready for rounds of nuclear transfer was 30 days. This period was made up of four stages: 2 days and 3 days for the initial establishment of the PDFF-2 working stock of primary cells, 20 days during which time gene targeting was carried out, and 5 days for the expansion of the clones and provision of multiple stocks for nuclear transfer. Between these stages cells were stored by cryopreservation.

Cryopreserved stocks of each cell clone were thawed, grown for 1-2 days then prepared for nuclear transfer as below.

Preparation of cells to act as nuclear donors.

Donor cells were plated at 2 X 10^4 cells per well of a 6 well tissue culture dish and cultured for 16-24 hours in BHK 21 (Glasgow MEM) medium supplemented with L-glutamine (2mM), sodium pyruvate (1mM) containing 10% fetal calf serum. At 24 hours the medium was aspirated and the cell monolayer was washed three times in medium containing 0.5% fetal calf serum. Cultures were then incubated at 37^{0}C in low serum medium (0.5%) for 2-5 days until they had exited the cell cycle as determined by staining for proliferating cell nuclear antigen (PCNA) in control cultures.

Cells were harvested by trypsinisation and stored in suspension in medium containing 10% FCS for 2-3 hours prior to use as nuclear donors.

Superstimulation of donor ewes and recovery of oocytes

Ewes to act as oocyte donors were synchronised with progestagen sponges for 14 days (Veramix, Upjohn, UK) and induced to superovulate with single injections of 3.0 mg/day (total 6.0 mg) ovine follicle-stimulating hormone (FSH) (Ovagen, Immuno-chemical Products Ltd. New Zealand) on two successive days. Ovulation was induced with an 8 mg single dose of a gonadotropin-releasing hormone analogue (GnRH Receptal, Hoechst. UK.) 24 hours after the second injection of FSH.

Unfertilised metaphase II oocytes were recovered by flushing from the oviduct at 24-29 hours after GnRH injection using Dulbecco's phosphate buffered saline containing 1.0% foetal calf serum (FCS) maintained at 37^0C until use.

Oocyte manipulation.

Recovered oocytes were maintained at 37^0C, washed in phosphate buffered saline (PBS) 1.0% FCS and transferred to calcium free M2 medium containing 10% Foetal Calf Serum (FCS), at 37^0C. To remove the chromosomes (enucleation) oocytes were placed in calcium free M2 containing 10% FCS, 7.5 ug/ml Cytochalasin B (Sigma) (Cytochalasin B is optional) and 5.0 ug/ml Hoechest 33342 (Sigma) at 37^0C for 20 minutes. A small amount of cytoplasm from directly beneath the 1st polar body was then aspirated using a 20 μM glass pipette. Enucleation was confirmed by exposing the aspirated portion of cytoplasm to UV light and checking for the presence of a metaphase plate.

Embyo Reconstruction.

Groups of 10-20 oocytes were enucleated and placed into 20μl drops of calcium free M2 medium at 37^0C, 5%CO_2 under mineral oil (SIGMA).

At 32-34 hours post hCG injection a single cell was placed into contact with the enucleated oocyte. The couplet was transferred to the fusion chamber (see below) in 200µl of 0.3 M mannitol, 0.1mM $MgSO_4$, 0.001mM $CaCl_2$ in distilled water. Fusion and activation were induced by application of an AC pulse of 3V for 5 seconds followed by 3 DC pulses of 1.25kV/Cm for 80µsecs. Couplets were then washed in TC199 10% FCS (the addition of 7.5 µg/ml Cytochalasin B to this medium is optional) and incubated in this medium for 1 hour at 37^0C, 5% CO_2. Couplets were then washed in TC199 10% FCS and cultured in TC199 10% FCS at 37^0C, 5% CO_2 overnight. Alternatively, the donor nucleus may be transferred by either manual or piezo aided injection or by any other chemical or physical means of producing cell fusion.

Embryo culture and assessment.

After the overnight culture period fused couplets were double embedded in 1% and 1.2% agar (DIFCO) (or any other suitable protective covering material) in PBS and transferred to the ligated oviduct of synchronised ewes. After 6 days recipient ewes were sacrificed and the embryos retrieved by flushing from the oviduct using PBS, 10% FCS. Embryos were dissected from the agar chips using 2 needles and development assessed by microscopy. All embryos which had developed to the morula/blastocyst stage were transferred as soon as possible to the uterine horn of synchronised final recipient ewes.

Example 3. Placement of a transgene at the COLIA1 locus in primary ovine fetal fibroblast cells by gene targeting.

The construct COLT-2 is shown in Figure 5. COLT-2 was generated by insertion of a MluI fragment containing the AATC2 transgene expression cassette into a unique EcoRV site situated at the 3' end of the IRES neo segment of COLT-1.

AATC2 consists of the 5' portion of the vector pACTMAD6 (described in patent application WO 99/ 03981), comprising the ovine β-lactoglobulin promoter and mouse cardiac actin 1st intron, linked to human α-1 antitrypsin cDNA and the 3' portion of the pMAD vector (described in patent application WO 99/ 03981), comprising the ovine β-lactoglobulin exon 7, polyadenylation site and 3 flanking region.

Having described the preferred embodiments of the present invention, it will be clear to those ordinarily skilled in the art that various modifications may be made to the disclosed details and embodiments and that such modifications are covered by the scope of the present invention.

CLAIMS

1. A method of preparing a somatic cell for nuclear transfer comprising modifying the genetic material of the somatic cell by a genetic targeting event.

2. A method as claimed in claim 1, wherein the genetic targeting event is homologous recombination.

3. A method, as claimed in claim 1 or claim 2, wherein the modification is inactivation, removal or modification of a gene; upregulation of a gene, gene replacement or transgene placement.

4. A method as claimed in any one of claims 1 to 3 wherein the genetic targeting event includes the artificial induction of gene expression or the induction of chromatin changes in the cell.

5. A method as claimed in any one of claims 1 to 4 wherein the genetic targeting event is facilitated by an agent which inhibits histone deacetylation or by expression in the cell of a factor which stimulates transcription at the target locus.

6. A method as claimed in any one of claims 1 to 5, wherein the somatic cell is an epithelial cell, or a fibroblast cell, or an endothelial cell.

7. A method of nuclear transfer comprising a method as claimed in any one of claims 1 to 6 and a method comprising transfer of the genetic material from the somatic cell to a recipient cell.

8. A method, as claimed in claim 7, wherein the transfer of the genetic material from the somatic cell, to a recipient cell, provides an animal embryo.

9. A method, as claimed in claim 7 or claim 8 further comprising the production of a totipotent or pluripotent cloned cell population.

10. A transgenic cell, suitable for nuclear transfer obtainable by a method as claimed in any one of claims 1 to 6.

11. A transgenic embryo or a transgenic fetus obtainable by a method as claimed in claim 8.

12. A method for preparing a transgenic animal, comprising causing an animal to develop to term from the embryo as claimed in claim 11 and optionally breeding from the animal.

13. A transgenic animal obtainable by the method as claimed in claim 12.

14. A transgenic animal as claimed in claim 13 which is a sheep, cow, bull, goat, pig, horse, camel, rabbit or rodent.

15. A transgenic animal which is bred from an animal as claimed in claim 13 or claim 14.

16. A method for obtaining a clonal pluripotent or totipotent cell population comprising culturing a cell line from a transgenic embryo or a transgenic fetus as claimed in claim 11.

17. A clonal pluripotent or totiptent cell population obtainable according to a method as claimed in claim 16.

18. A method for modifying the genetic material of a somatic cell while maintaining the totipotency of the cell, the method comprising a genetic targeting event.

19. A method as claimed in claim 18, wherein the genetic targeting event is homologous recombination.

20. A method as claimed in claim 18 or claim 19 wherein the modification is inactivation, removal or modification of a gene, upregulation of a gene, gene replacement or transgene placement.

21. A method as claimed in any one of claims 18 to 19, wherein the genetic targeting event includes the artificial induction of gene expression or the induction of chromatin changes in the cell.

22. A method as claimed in any one of claims 18 to 21 wherein the genetic targeting event is facilitated by an agent which inhibits histone deacetylation or by expression in the cell of a factor which stimulates transcription at the target locus.

23. A method as claimed in any one of claims 18 to 22, and a method comprising the transfer of the genetic material from the somatic cell to a recipient cell.

24. The use of artificial induction of gene expression or induction of chromatin changes in the genetic targeting of a cell.

25. The use, as claimed in claim 24, wherein the cell is somatic or non-somatic.

26. The use, as claimed in claim 24 or claim 25, wherein the genetic targeting is facilitated by an agent which inhibits histone deacetylation or by expression in the cell of a factor which stimulates transcription at the target locus.

27. The use, as claimed in any one of claims 24 to 26, in combination with the nuclear transfer of the genetic material of the cell into a suitable recipient cell.

28. A method of preparing a somatic cell for nuclear transfer, hereinbefore described with reference to one or more of the examples.

29. A method of nuclear transfer, hereinbefore described with reference to one or more of the examples.

30. A method of preparing a transgenic animal, hereinbefore described with reference to one or more of the examples.

31. The use of artificial induction of gene expression in the genetic targeting of a cell, hereinbefore described with reference to one or more of the examples.

Figure 1 COLT-1 gene targeting vector and the structure of the COLIA1 targeted locus

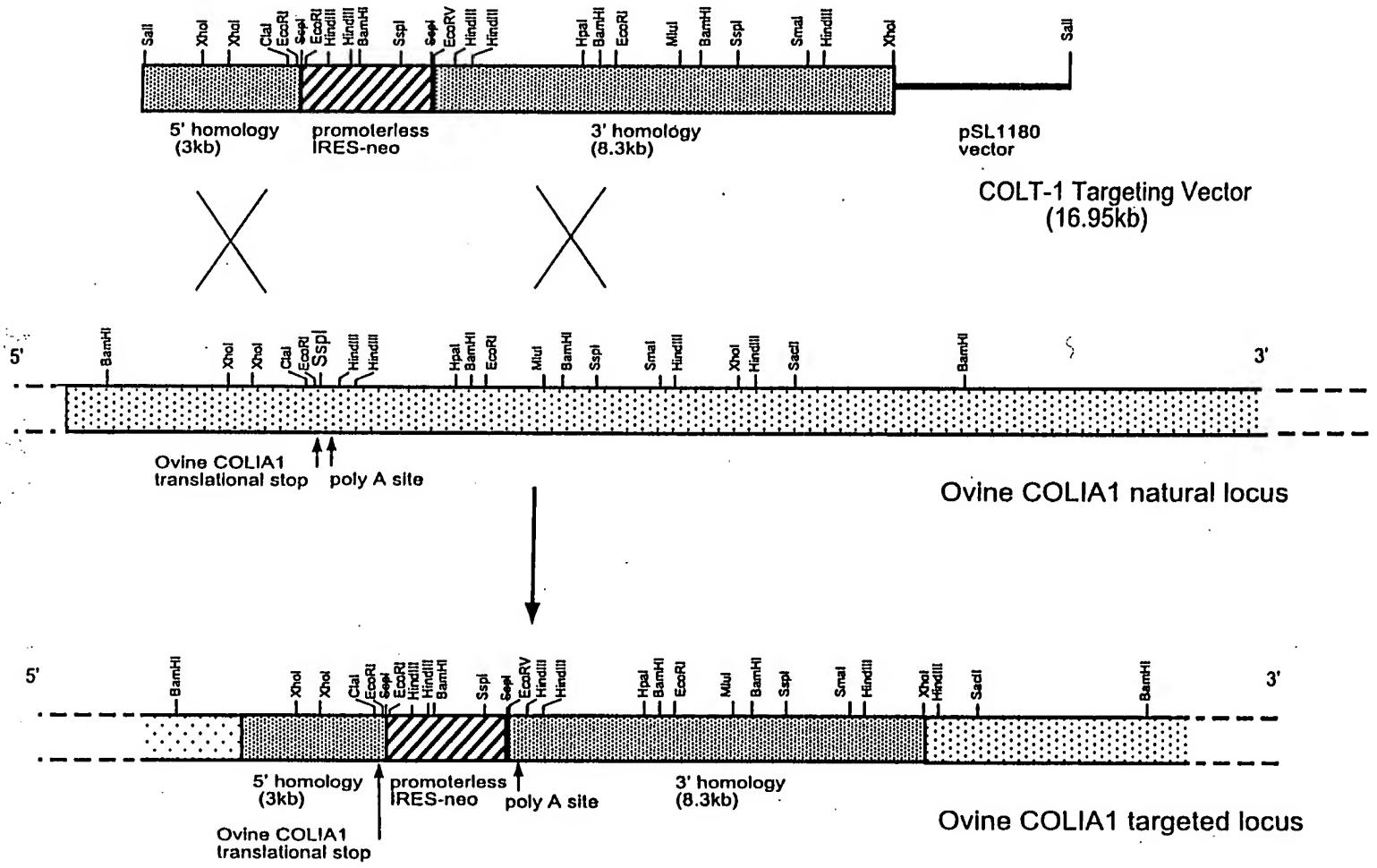
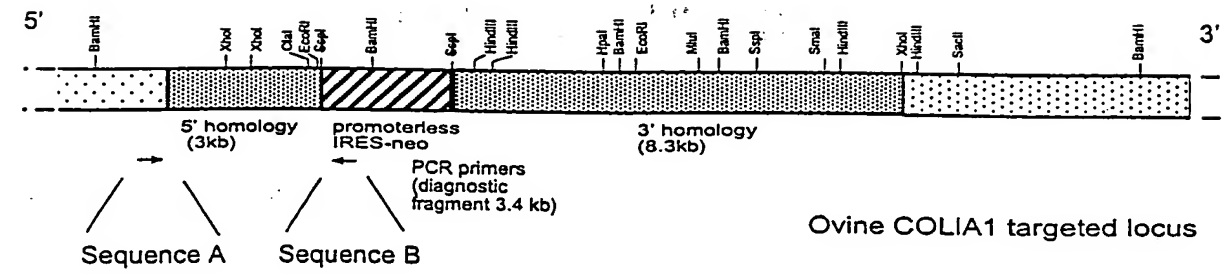
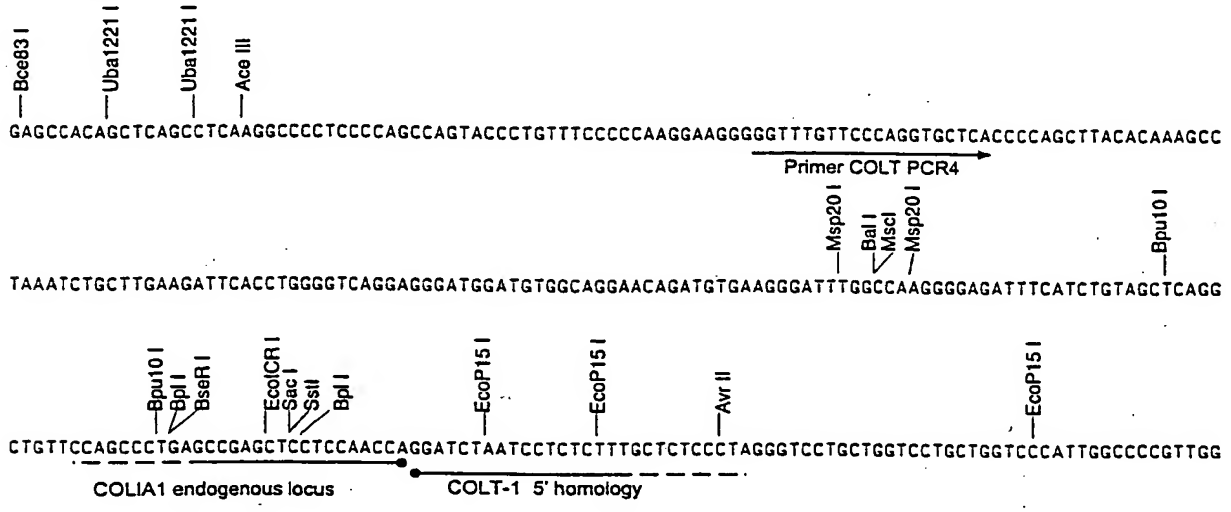


Figure 2. PCR screening strategy



Sequence A



Sequence B

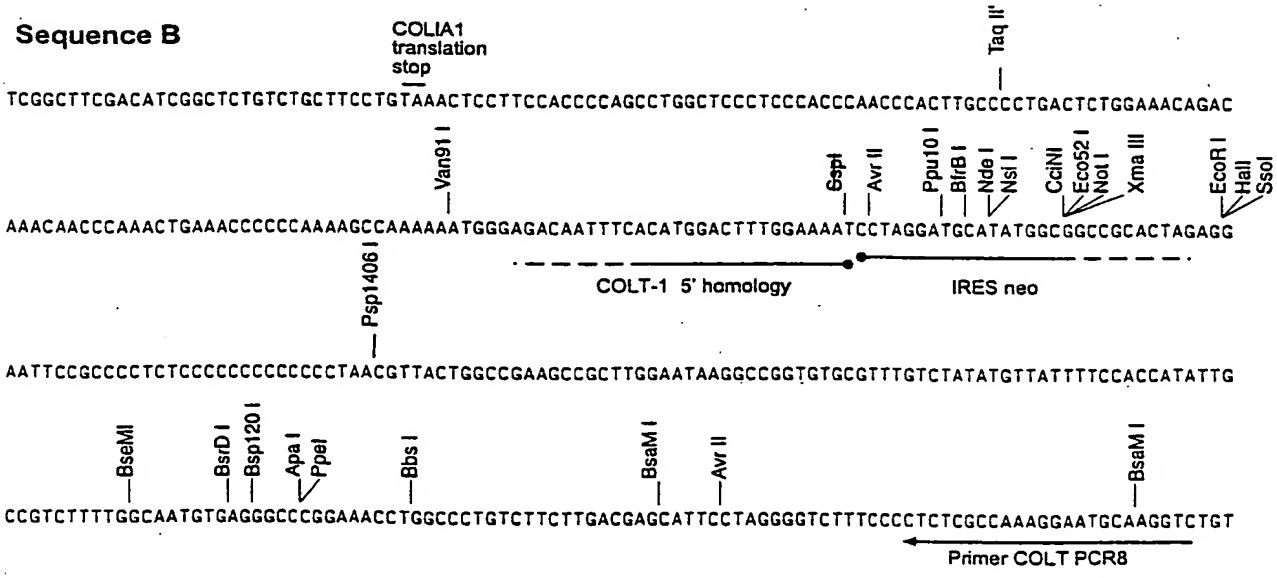


Figure 3. PCR screen of COLT-1 PDFF2 transfectants

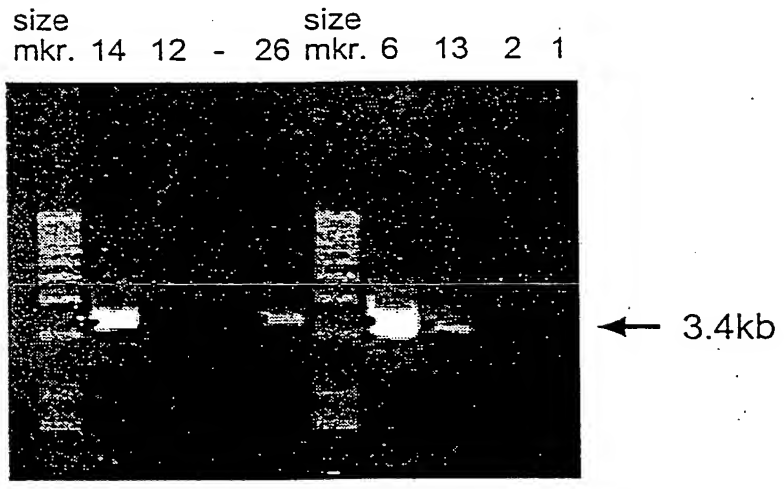


Figure 4. Sequence analysis at 5' junction of targeted locus

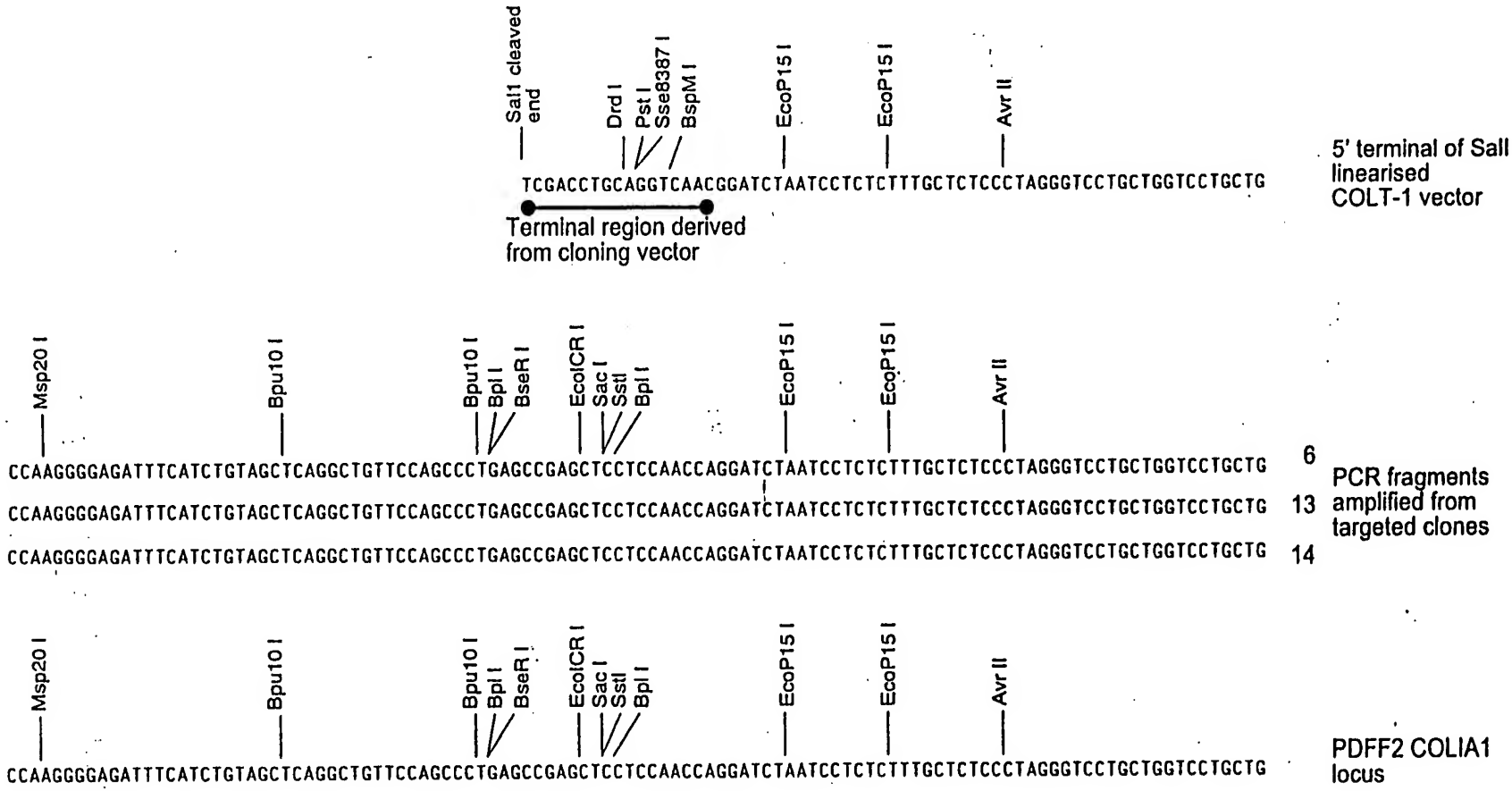


Figure 5. Construction of COLT-2 Transgene placement vector